IMMUNOLOGICAL ASPECTS OF DEOXYRIBONUCLEIC ACID AND CARCINOGENESIS

by

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INTRODUCTION

Physiological functions of the reticuloendothelial system (RES) have been established in terms of lymphopoiesis and lymph node regeneration, tissue repair, phagocytosis and cellular and humoral immunity. The concept is generally accepted that immunity, resulting from the participation of the RES, is specific in the sense that each antigen stimulates the formation of antibody for itself and no other antigen. However, the response of the RES in neoplastic disease has more general and less precisely defined characteristics. Thus, the role of the RES and the part it plays in cancer immunity should be carefully considered.

Attempts to apply immunologic theory and methods for a better understanding and treatment of cancer have been tried since Paul Ehrlich first worked in this field. Publications from clinical medical literature have indicated in some instances that improvement in cancer patients has resulted from treatment with antisera and vaccines; however, on the basis of scientifically acceptable data that has been published there is still no definitive immunologic basis for the treatment of neoplastic disease.

Applying the principles of immunology purely upon theoretical grounds, the successful eradication of cancer appears to favor the prophylactic approach rather than the treatment of an established

neoplasm. Southham (1961) reviewed the application of immunology to cancer and considered the experimental approach from four different aspects. These were:

1. "attempts to apply methods of specific immune therapy to the treatment of patients with advanced cancer;
2. passive immunization for the treatment of cancer;
3. stimulation of nonspecific host defense;
4. prophylactic immunization against cancer."

Southham concluded from this comprehensive survey that the first three statements have contributed little of practical significance in the treatment of cancer.

Most of the major effort in immunologic approach has been concerned with cancer therapy, even though the basic immunologic achievements have been with prophylactic immunity against disease. Transposing immunologic concepts to the prevention of cancer as with the prevention of bacterial disease would involve injection of the causative agent (neoplastic cells) into the host before the occurrence of disease or early in its course. Although the results of such efforts to date have not been promising in humans, this does not invalidate the approach. That is, in terms of immunologic theory one should consider the prospects for cancer prevention by immunologic methods. This in itself is a formidable task, since it necessitates a search for specific antigens in neoplastic tissue.

Rapport and Graf (1961) considered "cancer-specific" antigen to mean a constituent found exclusively in cancer tissue and, therefore, presumably "foreign" to the host. The premise is that such a substance would provoke an antibody response in the host which would be detrimental to the tumor. The antigenic substance would possess two qualities or attributes; first, that the distinctive constituent be antigenic in the host; and, second, that the antibody evoke a reaction injurious to the tumor cells.

An effort has been undertaken to more precisely define an antigen from cancer tissue. If such an antigen were available for immunization, competitive antigenic inhibition would be minimized and the concentration of antigen per unit volume injected could be increased. Since Avery et al. (1944) found that deoxyribonucleic acid (DNA) was the agent of bacterial transformation, much interest has been centered around its biologic activity. Interest has increased since Watson and Crick (1953) made their famous proposals about the structure of DNA and its mode of self replication. Nucleoproteins (DNP) and DNA are of interest with respect to cancer because they are important constituents of the chromatin structure in the cell nucleus. Considering the morphological irregularities in chromatin structure of the nucleus of benign and malignant tumors, there appears to be a definite sequence of nuclear abnormalities (Koss and Durfee, 1961). This consistent histological finding of abnormal chromatin in the nucleus of malignant cells suggest the possibility that DNP or DNA may be chemically or sterically different and antigenically distinct from its normal counterpart.

To test the hypothesis that DNP and DNA are antigenic, and that these substances derived from cancerous cells might induce resistance to neoplastic disease, the following investigations were undertaken: (1) DNP and DNA were prepared from EAT and S37 ascites tumor cells. (2) The prepared material was used to immunize mice subsequently challenged with viable tumor cells. (3) Specificity of reaction was tested by challenge with an ascites tumor other than that used for immunization.

REVIEW OF LITERATURE

It wasn't until the beginning of this century that scientists began to design experiments to fit a particular set of circumstances. Before this time they were content to make casual observations of what was either present or occurred spontaneously in nature. Admittedly many of the experimental tools were lacking at this time but the scientific approach was also unimaginative by current standards. Emergence from this era to the present one came about slowly but as attitudes changed so did the lines of endeavor.

Forerunner of experimental studies of carcinogenesis was the finding of Percival Pott (Stewart, 1959) who published a description in 1775 in "Chirurgical Observations" of a carcinoma of the skin, cancer scroti, occurring in chimney sweeps. His observations traced the causative agent to the soot to which the chimney sweeps were constantly being exposed. It wasn't until a century and a half later that Yamagiua and Ichikawa (1914) using coal tar were able to confirm that carcinogens did exist and the carcinoma of the skin could be induced in rabbits by repeated application to the skin for sufficient lengths of time. Since this first successful induction of tumor by a carcinogenic agent, many experimental models have been tried in efforts to obtain insight concerning the mechanism of this disease, its induction as well as its prevention.

Since many of the terms used in the literature in dealing with transplantable tumors are conflicting, it would be well to define certain terms pertaining to this investigation.

Neoplasm as defined by Ewing (1940) is an uncontrolled new growth of tissue. The term "tumor" is applied to any neoplasm, benign or malignant. A cancer is a malignant neoplasm which eventually kills the host. Carcinoma is a malignant tumor of epithelial origin; sarcoma is one which arises in mesenchymal tissue. A teratoma may be either benign or malignant and is reproduced from ectoderm, entodern and mesenchymal tissues. For the purpose of this investigation, carcinoma, tumor and cancer will be used synonymously.

The term ascites tumor has come to denote a process in which, following inoculation of tumor cells into a body cavity, the tumor cells multiply, and there is effusion of fluid containing a suspension of neoplastic cell (Stewart et al., 1959).

Snell (1959) suggested the following terms in his discussion of transplantable tumors.

An inbred strain of animals is one resulting from matings of brothers and sisters for 20 or more consecutive generations. Some increase in homozygosity occurs after the twentieth generation, but for most purposes inbreeding for 20 generations is adequate. An equivalent increase in homozygosity is produced by parent-offspring matings, provided that where consecutive parent-offspring matings are made the cross is always to the younger of the two parents.

An autotransplant or autograft is one in which an individual is

grafted with its own tissue.

An isotransplant or isograft (isogeneic graft) is one in which tissue from an individual of an inbred strain is grafted to another individual of the same inbred strain.

A homotransplant (allograft or allogeneic transplant) is a transplant between individuals of the same species but of different genotypes. Typically, it is a transplant from an individual of one inbred strain to an individual of a second inbred strain.

A heterotransplant (xenograft or xenogeneic transplant) is a transplant between different species, as from rat to the mouse.

More recently, based on Gorer's (1960, 1961) suggestions, Russel and Menaco (1964) tabulated a desirable terminology of tissue transplantation which is shown in modified form in Table I.

An orthotopic graft is one which is placed in proper anatomic position, i.e., skin to skin, kidney to renal fossa with proper anastomosis at renal pedicle.

A heterotopic graft is one which is in an unnatural position, eg., adrenal tissue in peritoneal cavity.

It is apparent from Table I that Snell's suggested terminology should now be modified to replace the terms given in parentheses.

Other conflicting terms among investigators involve description of the type of immunity elicited by different tumor host systems; these are: autoimmunity, isoimmunity (isogeneic immunity), and homograft (allogeneic) immunity. These terms are defined by Hirsch (1958)

TABLE I.

Tissue Transplantation Vocabulary

Term and Adjective	Older, Less Correct but Common Term	Meaning
Autograft-autologous	autograft	Donor of graft tissue is also recipient
Isograft-isogeneic	isograft	Grafts of tissue between inbred strains or "identical" twins
Allograft-allogeneic	homograft	Grafts of tissue between members of "wild" strains i.e., homologous members of species
Xenograft-xenogeneic	heterograft	Grafts between species

as follows: autoimmunity is "immunity elicited in an animal against his own normal or tumor tissue." The host then is referred to as the autochthonous host. Isoimmunity (isogeneic immunity) is defined as "immunity elicited in a member of an inbred strain against normal or tumor tissue of another member of the same inbred strains"; and homograft (allogeneic) immunity is defined as "immunity elicited in an individual against normal or tumor tissue of a genetically different individual of the same species, or that elicited in animals of one inbred strain against normal or tissue of members of other inbred strains of the same species." The terms given in parentheses are used hereafter.

The concept that cellular antigens specific for cancer may exist has been formulated by several investigations: Hauschka (1952) and more recently Southham (1960), Green (1961), Law (1961), Hirsch (1962), and McKhann (1965). Although, as these authors have indicated, greater insight into the mechanisms involved in cancer immunity is constantly being achieved, it remains difficult to identify tumor specific antigen.

Immunological reactions that are attributable to antibody produced by the host against its own tumor should bear some relation to the presence of "foreign" constituents in cancer. An experimental design for detection of this circumstance is as follows: a cancer tissue homogenate or a subfraction is injected into the test animals and the animals are then either subjected to challenge by the original

tumor or standard immunologic procedures should be tried to demonstrate an antibody to the test material. There is considerable evidence that injection of cell homogenates or subfractions is followed by formation of antibodies. Formation of antibodies directed against cellular antigens has been demonstrated by Gorer (1938, 1942, 1948) and Goldner et al. (1959). Harris et al. (1954) and Snell (1960) concluded from experiments involving immunization of mice with homogenates of thymus glands that cellular immunity is of lymphoid origin. Law (1961) in a review of immunologic aspects of carcinogenesis suggested that in transplantation immunity the reaction is apparently provoked by nuclear antigens.

Makari (1962) presented evidence that carcinoma antigens possessed a high degree of specificity. If such antigens are unique in cancer tissue, then there is clearly the possibility for prophylactic immunization. If we are to accept the hypothesis that some constituents of cancer cells are antigenically different from their normal counterpart, then the need to search for specific antigen (s) in neoplastic tissue is evident.

Landsteiner (1946) supported this conclusion with the following statement: " the experience of poor and wanting antigenicity of animal nucleoproteins which are composed of nucleic acids and histones or protamines is intelligible since, as evidence hitherto existing would indicate, the protein components have no appreciable antigenic capacity. Bacterial and virus nucleoproteins, on the other hand, were

found to induce antibody production. In view of positive results with some nucleoproteins of animal origin it would be worthwhile to resume the subject with the use of well purified and unaltered preparations."

I. RESISTANCE TO TUMOR TRANSPLANTS

A. Antigenic Basis of Resistance to Transplants.

Although the problems encountered in the investigations described in this thesis do not directly pertain to other well established concepts of the antigenic basis of resistance to tumor transplants, no discussion of tumor transplantation would be complete without citing some of the concepts established by Gorer and his colleagues. Gorer (1938, 1942, 1948, 1962) has shown that isoantibodies could be produced in certain tumor-host combinations in mice.

Gorer's (1938) original concept of antibody formation was traced to specific gene differences between donor and host. Using inbred strain A and C57BL mice and employing a leukemia specific for the A strain animals, it was found that by challenging strain C57BL with leukemia cells of A strain that agglutinins for A strain erythrocytes developed. Since the agglutinins could be absorbed by the A strain tumor cells, it was concluded that strain A tumor cells and erythrocytes shared a common antigen. This antigen designated as antigen II could also be demonstrated by injecting A red cells into rabbits and

producing antiserum. This antiserum would specifically agglutinate A red cells when absorbed with C57BL red cells.

Later experiments by Gorer and Amos (1956) showed that it was possible to obtain protection by passively immunizing C57BL mice with antiserum obtained by immunizing foreign strain mice with leukemia EL4. Absorption *in vitro* with normal C57BL liver failed to deplete the passive immunity. However, when the same antiserum was absorbed with EL4 cells, there was a marked reduction of protective activity. In order to establish the specificity of this antigen, foreign strain mice were immunized with normal tissues and mammary carcinoma of the C57BL, and these mice failed to provide any passive immunity as compared with antiserum prepared with EL4 cells. Gorer *et al*. (1962) tried to establish the identity of the antigen which they designated as X. Using a series of leukemic cells from different sources, three distinct X antigens were found. One of these antigens cross-reacted with the other two X antigens. They concluded that these antigens may be variations of normal elements found specifically in leukemic cells, or that they may represent marked quantitative antigenic differences between leukemic and normal cells of similar genetic origin.

Kidd (1938, 1940a, 1940b) using antisera obtained from rabbits with Brown-Pearce tumor which had regressed, was able to demonstrate complement-fixation with tumor antigen. Although this finding further substantiated Gorer's work, the antigen did not appear to be

specific. Absorbing the antisera with both normal and with Brown-Pearce tissue extracts, it was shown that two different antibodies were present: (1) an antibody that reacted with both normal tissue and tumor extracts; and (2) an antibody specific for tumor extracts.

From these investigations it appears that tumor cells per se contain a specific substance or substances that are antigenically demonstrable. The results, although conflicting in some instances, indicate that tumor cells do contain specific antigens and that they can elicite an antibody response. This antibody can be demonstrated not only in vitro by agglutination and complement-fixation procedures but also in vivo by passive immunization.

B. Enhancing Effect

The main purpose of this investigation was to study means of increasing resistance to tumor cell implantation. However, in other circumstances it would be profitable to increase the survival of tumor tissue and without question one must find practical procedures to induce the survival of tissue grafts from donor to host. The rejection or acceptance of tissue whether it be normal or neoplastic is related basically to the total problem of transplantation. Superficially both processes appear to be a opposite extremes; however, from an immunological basis, the processes must be intimately related to each other.

Kaliss (1958) defined immunologic enhancement as "...the success-

ful establishment of a tumor homograft and its progressive growth (usually to death of the host) as a consequence of the tumor's contact with specific antiserum in host." The primary requisite for growth of a tumor that would be normally rejected is conditioning the host; this may be done either by active or passive immunization. Conditioning the host is carried out by pretreating animals with tissue, not necessarily from tumor tissue itself but tissue that must share certain genetic factors with the host's tissues. In other words, the effort is made to induce "tolerance." Kidney, liver, and spleen have been found to produce enhancement as well as the tumor itself (Kaliss and Snell, 1951). The pretreated animal will die from progressive tumor and the controls will reject the transplant and survive. This phenomenon therefore has been called "immunological enhancement" to distinguish it from other experimental conditions that may cause rejection or survival of the graft.

Kaliss (1962, 1965) considering the mechanisms of enhancement found that more uniform results could be produced by passive rather than by active immunization. This is expected, as animals, even if inbred, vary in their response to immunologic stimuli. If active immunization is carried out, killed tissue is more effective for enhancement as it does not produce an efficient cellular response, but does induce a good humoral response. Live tissue produces a "second set" response and rejection rather than enhancement. Tissues used for the enhancement effect generally have been killed by freeze-drying in order to prevent extensive denaturation.

The enhancement effect is highly specific. Kaliss and Newton (1949) have shown that non-mouse tissue does not produce enhancement of mouse tumor homografts. Snell, et al. (1953, 1954, 1955) indicated that in order for tissue cells to be effective in enhancement they must possess certain histocompatiblity genes, and that the isoantigens resulting from these genes must be common with the tumor. He designated these genes as H-1, H-2, and H-3, and believed them to determine the susceptibility and resistance to transplants.

Kandutsch (1956) has presented evidence from chemical studies that the enhancing substance contains both mucopolysaccharides and protein. Experimentally, at least in some instances, this appears to be substantiated. Generally it is conceded by most investigators that the active antigenic portion of the ABO system are blood group substances composed of mucopolysaccharides. As previously stated in Gorer's original work, since there was a common antigen between leukemia cells and A cells, the antigens are mucopolysaccharides in nature.

Although the exact mechanism of immunologic enhancement is still open to speculation, it is generally agreed that primarily the reaction is due to contact between graft and antigraft antibody. Kaliss (1962) has stated that various investigations have attributed the mechanism of enhancement to: (1) "afferent" blockage of the immune stimulus; (2) "central" blockage of the immune response; (3) "immunoselection" of insusceptible cells in a mixed antigenic population of tumor cells;

(4) on some alteration in the enhanced graft that makes the graft resistant to the host's immune response. It was Kaliss' belief that immunoselection has been ruled out and that there is some evidence against the blockage hypothesis. He supported this by stating that enhancement of Sarcoma I grafts in C57BL mice would be obtained by injecting "enhancing" antiserum one week after tumor inoculation, when the homograft rejection reaction is maximum.

Whatever the mechanisms that contribute to immunologic enhancement, it must be concluded from this review, that one can still only speculate concerning the reactions that are involved. It appears then, that an antigen-antibody reaction occurs and that specific antigen(s) exist. Whether this antigenic quality is due to gene differences expressed as specific substances within the cell or in some other manner remains unknown. We can only hypothesize that within the cell certain antigenic differences exist and that definition of this substance or substances await further experimentation.

C. Increased Resistance.

Immunology with all of its ramifications is still primarily concerned with augmenting resistance to disease. The goal of an immunization procedure is to prevent or ameliorate the course of disease whether it be bacterial, viral or neoplastic in nature. Of course, the greatest success in inducing increased resistance has been with bacterial and viral diseases, in which cases the antigenic com-

ponents are more precisely defined. However with cancer, the antigenic components of the cell have less definition, and methods of testing for antibody response are not often revealed by classical immunological methods. One of the major purposes then of cancer investigation is to seek a "specific" antigen and to augment resistance in the host.

All investigations of malignancy to date have failed to reveal any antigenic qualitative distinction for the mobilization of defense mechanisms against this disease. Antigenic differences noted by workers in cancer immunology have yet to be resolved. However, on the basis of some experimental evidence the possibility exists that certain neoplastic tissues do possess antigens and that these antigens are qualitatively distinct. Progress in cancer immunology has been reviewed by Woglom (1929, Hauschka (1952), Foley (1952), and more recently by Milgrom (1961) and Southham (1965). The studies cited in these reviews have served to emphasize the specificity of induced immunity to neoplastic disease.

If we are to accept the hypothesis that some constituents of cancer cells are antigenically different from their normal counterpart, we should first reconsider and evaluate some of the present-day ideas. Southham (1961) stated that regardless of the fundamental efficacy of defense mechanisms against cancer, the patient who has acquired cancer must be an individual who has failed to respond to the stimulus of his own cancer. In effect this is certainly true,

but on the other hand, if we compare different states as exhibited in bacterial infectivity and immune response we could derive the same conclusions with regard to infective disease.

There is considerable evidence that fragmented cells and extracts of tumor tissue used as antigens are accompanied by formation of antibodies. Marshak and Erf (1941) immunized a small percentage of mice against lymphoma by first giving subcutaneous injections of fragmented lymphoma cells and isolated lymphoma cell nuclei. Goldfeder (1945) immunized rats against lymphosarcoma using similar tumor tissue from irradiated rats. Aptekman et al. (1946, 1949) also were able to immunize rats against transplanted and induced sarcomas; however, these investigators used alcoholic extracts of the tumors.

Quantitative studies of Old et al. (1961) indicated a wide range of antigenicity of induced tumors, from strongly antigenic to no demonstrable antigenicity. Depression of the antibody forming system after carcinogenesis should increase tumor incidence, particularly with the common therapeutic procedures which depress antibody production. Furth (1963) has implied that when antigenic cancer cells attain such a mass as to constitute an antigenic excess, there is created an "immunologic paralysis," which inhibits effective circulating antibodies. It follows then that continued depression of immunologic capacity, during which the tumors attain a sizable mass, can create a fatal immunologic paralysis.

Recently several investigators have tried to search for the presence of antibody individuals in whom tumor arose. In the studies of Graham and Graham (1955) and Marcissov and Abelev (1959), antibody was detected in the patient's serum by the method of complement-fixation. Makari (1962) demonstrated antibody in cancer patients by the Schultz-Dale technique, using sera from subjects with and without neoplastic disease. Grace and Kondo (1958) based their results on skin sensitivity to intradermal injection of a tumor extract and passive transfer using the patient's serum. Each of these studies indicate that antibody was found in only a limited number of cancer patients. For example, in the studies of Graham and Graham, tests were positive in only 12 out of 18 patients; in those of Makari 90.2% were positive in subjects with carcinoma and 25% positive in normal individuals; Grace and Kondo reported positive findings in only eight selected patients. In the study of the Grahams, it was shown that the "antibody" may be individual-specific, which makes it difficult for further study. Makari did not believe that 25% false positives distracted from his test, as it was an advantage as a screening procedure.

Rapport and Graf (1961) concluded from their experience that some antigens may be too specific to be utilized for immunization, but genetically controlled antigens that make tissues "individual-specific", i.e. nontransplantable, are also present in cancer tissue. Makari (1962) on the other hand presented evidence that carcinoma antigens have a high degree of specificity. If such antigens are unique

in cancer tissue, there is clearly a possibility for prophylactic immunization.

As indicated from the discussion on immunologic enchancement, there is no longer a question whether or not cells can proliferate in heterologous hosts if no immune forces come into play. A question that is foremost in tumor investigations is concerned with factors necessary for increasing resistance of the host against viable tumor cells. Generally speaking the use of killed cells as a vaccine has been found to be unsatisfactory. Most of the successful immunization procedures have been carried out using live cells or cells altered by x-irradiation. It could be hypothesized from this that methods used for killing tumor cells have destroyed their antigenic capabilities and are no longer recognized by the host. From the work of Medawar (1954) it is apparent that the animal host reacts to different homologous tissue as it does to bacteria and foreign proteins. Thus, the stability to preparatory treatment of the materials used in immunization would determine whether they still retained their antigenic qualities.

Goldfeder (1945) and Donaldson and Mitchell (1959) have shown that x-irradiated tumor cells could induce resistance to later injection of viable cells. However, Goldfeder (1942) demonstrated that sarcoma 180 cells conferred active immunity if the cells were irradiated <u>in vitro</u> with 4000 - 5000 rad but not 60,000 rad. Hill and Marcus (1960) and Baillif (1960, 1964) were able to increase resistance by injecting viable Ehrlich ascites tumor cells intraven-

ously but failed to induce immunity by using cells killed by formalin. These reports indicate that the antigenic capabilities of cells are easily destroyed, but that the use of live cells or cells but slightly altered by x-irradiation increased resistance to subsequent challenge.

II. TRANSPLANTABLE TUMORS

A. Induction.

Since Yamaguia and Ichikawa (1914) induced carcinoma for the first time in the skin of rabbits by painting with tar, many experimental models for the study of carcinogenesis have been introduced. Neoplasms of many different types have been induced and transplanted in a variety of animals. Snell (1959) has compiled a list of transplantable neoplasms, which conveys some idea of the great variety of tumors and the number of different organs that are involved. The list includes spontaneous tumors and also tumors induced by chemical and physical means. Stewart, Snell, Dunham, and Schlyen (1959) have also compiled a monograph describing transplantable and transmissible tumors of animals in detail.

Since Foley (1953) first demonstrated that subcutaneous sarcomas induced by 3-methylcholanthrene in one member of an inbred strain of mice were antigenic in isologous hosts, chemically induced tumors have gained considerable popularity in the study of the immunology of tumorgenesis. This was later substantiated by Prehn and Main (1957) and Klein et al. (1960) who were able to induce a tumor-

specific immune state in isologous mice or even in the autochthonous host. Prehn (1960) was also able to demonstrate similar results with another polycyclic hydrocarbon, dibenz[a,h]anthracene. However, in physically induced tumors, such as tumors induced by implantation of millipore filters, an immune mechanism is less well defined, (Prehn, 1961, 1965).

Recently extensive use of ascites tumors has been made in the study of tumorgenesis. This has been made possible by conversion of solid tumors to ascitic form (Klein, 1955) and New York Academy of Science conference on ascites tumors, Hauschka (ed.) (1956). The use of ascites tumors as compared to solid tumors has several advantages: (1) cell counts and LD_{50} values can be readily ascertained; (2) death is rapid; (3) transfer and propagation of the tumor can be readily accomplished. The disadvantages of using this type of tumor are that the course of the disease resembles an overwhelming infection, the existence of immune mechanisms can generally be studied only by resistance to challenge, and in most cases the tumor is nonspecific in the sense that it will grow in any strain of mice.

The use of solid tumors offers a better means to evaluate the immune response, particularly when using autochthonous hosts, since the tumor can generally be completely excised and then reinoculated as a challenge dose. The course of death, however, is markedly prolonged, individual cells in suspension are difficult to obtain, and LD_{50} values are therefore difficult to determine. The technical

difficulties in measuring the number of cells in the inoculum and the laborious task of mouse to mouse transfer by trocar implantation of tumor cells are evident.

The different routes of inoculation, size of inoculum and age of the tumor used have a profound effect on success of transfer of any experimental tumor. This appears to be true whether using an ascites or solid tumor. Oshiro (1963) demonstrated a markedly different LD_{50} value using various routes of challenge with S-37 and EAT ascites tumor cells. Oshiro's results indicate, in the case of EAT cells, that one virulent tumor cell can induce lethal disease via the intraperitoneal route. In contrast to this highly "infectious" route, mice were found to tolerate an intravenous injection of 10^6 tumor cells without evidence of mortality. The LD_{50} values of S-37 tumor, via the subcutaneous and intravenous routes were 1.25×10^5 and 3.2×10^3 cells respectively. These findings vividly demonstrated not only the different dose responses with the tumors used but also the significance of the route by which the challenge dose was administered.

The number of cells as well as the age of the donor tumor that is transplanted is important to success of the transplant. Zahl and Drasher (1947) found that seven-day growths of sarcoma 180 (Crocker tumor) contained mostly viable cells while two and twelve-day growths contained many necrotic cells. This observation was checked by measuring growth rates of the seven-day old tissues as compared to the two and twelve-day old tissues.

B. Transformation.

Transformation is the change that occurs in transplantable tumors during repeated transfer. The changes that occur are not only in the direction of decreased host specificity and increased virulence but also morphologic changes of cell types. The tumor cells that an experimenter uses for a particular set of experiments may be different from those which were employed previously and from those employed at a later date.

Miller and Taylor (1948) using a strain of A line leukemia cells, after many transfers, were able to decrease the time of death from fifteen to four days. MacDowell (1946) was able to show in line M-liv leukemia cells an abrupt acceleration of growth during the seventeenth and eighteenth transfers. During these transfers the the time of deaths decreased gradually from 45 days to 5 days and then remained stable for many transplant generations. This suggested that a biologic adaptation occurred on the part of the tumor cells.

Morphological changes have also been noted in tumors after many transfers. Stewart et al. (1947) noticed that in isogeneic transfers of pulmonary tumors in mice, a fibrosarcomatous transformation occurred and that many of the tumors retained mixed carcinomatous and sarcomatous characteristics through a number of generations. A few of the tumors ultimately became pure sarcomas. Whether or not the observed changes were due to an unrecognized mixture to tumor cell types and were merely a case of selection is open to

speculation. However, Cooper et al. (1944) noted that there was a loss of keratininzation after 20 subcutaneous passages of a squamous cell carcinoma.

Variation of present day lines of tumors also becomes evident from the difference in nutritional requirements observed in tissue culture. Puck and Fisher (1956) have reported that with Hela cells, variants were produced that show different nutritional requirements.

Furthermore, Sacks and Gallily (1955) studying the chromosomal number of ascites tumors, state that diploid tumors have a greater degree of strain specificity than do tumors characterized by more than a normal number of chromosomes. Stewart et al. (1959) have reported a variation in the modal number of chromosomes from 45 to 80 in EAT ascites tumor. Oshiro (1963) has described this same tumor as having a modal chromosome number of 60 with a range from 41 - 83.

III. RETICULOENDOTHELIAL SYSTEM

The reticuloendothelial system (RES) is composed in part of reticular cells, endothelial cells, fixed and wandering macrophages, and microphages of the blood. This system was first described by Aschoff (Bloom, 1962). The primary functions of the RES are to rid the body of worn out cells, foreign debris, and particles, animate or inanimate. Metchnikoff realized that although these cells were dissimilar under various physiological conditions, their protective function was the same. He called these cells macrophages and related

them to the defense mechanism in inflammation and immunity. Bloom (1962), thus, has termed the original RES, the macrophage system.

A. Humoral Immunity.

Despite the fact that a voluminous literature has accumulated concerning the role of humoral immunity in graft and tumor rejection, the subject remains controversial. Classical humoral antibodies to some experimentally induced tumor homografts are commonly produced; however, the participation of these antibodies in tumor rejection has been demonstrated only in a limited number of tumor-host combinations. Hericourt and Reichet (1895) were among the first to suggest that humoral antibody might be used in the treatment of an established neoplasm. Then, as now, host defense mechanisms to neoplasia were still to be delineated.

The immune state may be acquired actively by introducing a foreign tissue into a suitable animal or it may be acquired passively by the introduction of antibody prepared in a homologous animal. Reactions of heterologous immune serum against homologous normal tissue or tumor grafts have been demonstrated by the cytotoxic activity of such antibody. Because this antibody is better demonstarted in allogeneic (homograft) or xenogeneic (heterograft) experiments, it has been used extensively in the search for tumor specific antigens.

The cytotoxic activity of rabbit immune gamma globulin prepared against Krebs ascites tumor has been shown by Green et al. (1959).

In vitro exposure of these cells to immune gamma globulin plus complement resulted in a loss of free amino acids, 30 - 60% cell protein and 90% of intracellular potassium. Ultrastructural studies by Goldberg and Green (1959) of the same system revealed a distinct alteration of the cellular membrane. When complement was subsequently added to the system, severe damage to the matrix, mitochondria, and endoplasmic reticulum was observed.

Bjorklund *et al*. (1958) prepared antiserum in a horse by subcutaneous injections of crude antigens from pooled human carcinomas over a period of several years. The rational for this immunization procedure was that an antigen mutually shared by cancers might eventually predominate over other antigens that are common to normal and cancer cells. Bjorklund and Bjorklund (1957) employed absoption methods and showed that the cytotoxic anticancer antibody was specifically directed towards a cancer specific antigen. These tests involved a comparison of reactions between tumor or normal cell extracts and absorbed antibody. A method of assaying this antitumor antibody was described by Bjorklund *et al*. (1961). The assay involved the cytolysis of tumor cell cultures by the prepared antiserum.

Southham and Tetsuo (1963) were able to demonstrate isoantibodies to human cancer cells in healthy recipients of cancer allogeneic (homo) transplants. Such transplants of nine cancer cell lines and two normal cell cultures were made in 20 healthy male volunteers. Sera collected before and after implantation were studied using the

tanned erythrocyte agglutination technique. The data demonstrated that the cancer cell lines contained an antigen or antigens in common which were foreign to the transplant recipients and were rarely detectable in the normal cell lines and normal tissues which were studied. These results are consistant with the concept that a humoral type of immunity can be demonstrated against cancer specific antigens.

Old et al. (1963) were also able to demonstrate the cytotoxic effect of antiserum on tumor cells; however, the results of their investigations did not reveal a common antigen such as was shown in Bjorklunds' experiments. Viable cells from leukemic spleens induced by Friend virus in Swiss mice were injected into BABL/c mice to produce antisera. The cytotoxic activity of the antisera was restricted to leukemia induced by Friend virus but was inactive against three other mouse leukemias.

Passive transfer of tumor immunity with sera has also been demonstrated. Lindeman (1964) demonstrated that serum or plasma taken from mice 3 to 8 weeks after viral oncolysis of EAT could transfer immunity to challenge by the original viable tumor. The technique used was to mix serum and tumor cells and to inoculate the mixture ip within one hour of preparation. Tyan and Cole (1963) were also able to demonstrate passive transfer of immunity. They presented data showing that the initial response to an allogeneic skin graft would be inhibited significantly in sublethally irradiated mice but that the response was essentially restored by passively transferred specific antisera.

B. Cellular Immunity.

As previously mentioned there exists diversity of information and opinion concerning cellular reponse to tumor and normal tissue graft implantation. Recently Prehn (1965) has seriously questioned the role of humoral antibody and the part it plays, if any, in tumor immunology. Prehn believes that immunity as exhibited in neoplasms is primarily a cellular phenomenon. However, many investigators ascribed to both humoral and cellular factors responsibility for acceptance or rejection of neoplastic implants.

An immune type of reaction mediated by cells was first indentified by Landsteiner and Chase (1942). They demonstrated that a delayed type of sensitivity to simple organic compounds could be transferred by cells and not by serum. Billingham et al. (1954a, 1954b) have named this state of immunity "adoptive" immunity, that is, the immune state is transferred from one animal to another by immunologically activated cells. They showed that skin homografts on tolerant hosts would be activated immunologically after transfer. The skin homografts were accepted by the host, but could be subsequently rejected by intraperitoneal inoculation with lymph node fragments from mice of the allogenic strain. This concept of immune status mediated by cells has been further substantiated by Algire et al. (1955) who demonstrated that the reaction could be blocked by using membranes impermeable to cells placed intraperitoneally containing immunologically activated cells.

Baker et al. (1962) concluded from their investigation of the immune destruction of Sarcoma I ascites tumor in the peritoneum of the C57 BL/LK mouse that such destruction was due largely, if not entirely, to the contact activities of the peritoneal macrophages. They stated that the findings which contribute most to this hypothesis are as follows: (1) among host cells of the ascites the macrophages were the only cells that showed a marked increase in concentration before the onset of tumor destruction; (2) the peritoneal macrophages were the only host cells that displayed mixed affinity for the tumor cells; (3) passive transfer of tumor immunity was accomplished with macrophage-rich ascites from actively immunized animals, but not with the sera, cell-free ascites, and extracts of spleen and peritoneal cells from such animals; (4) when tumor cells were enclosed in cell-impermeable millipore chambers and implanted in the peritoneal cavities of actively immunized animals, the cells remained viable for at least three weeks. Lindermann (1964) further supported this hypothesis that cells from the immune host can confer tumor immunity with the following experiment: mice which had survived viral oncolysis and were challenged six weeks later with 10^6 EAT cells were killed five weeks after challenge. One group of normal mice received 5×10^6 and another group 5×10^7 immune spleen cells/mouse intraperitoneally. After 24 hours the test and control groups were challenged with 10^4 EAT cells. All the control mice died, whereas all mice pretreated with cells survived.

This section may be summarized by stating that significant evidence exists to show that cells, both lymphocytes and macrophages, may be of prime importance in the rejection of certain tumors and allogenic skin grafts.

IV. DEOXYRIBONUCLEIC ACID TUMOR ANTIGEN

Early investigators of cancer immunity dealt mainly with attenuated cells, tissue homogenates and cell extracts. From the review that has been presented, it is apparent that the search for the elusive specific cancer antigen(s) still continues. These investigations, frequently yielding conflicting results, have a common purpose, that is the characterization of a cancer antigen and subsequent eradication of cancer through the use of immune procedures. "Supposing that a distinctive constituent has been detected in cancer tissue, how shall we proceed? We think the answer is straight forward: find out what it is, determine its properties, then make it available for study in various experimental situations. These objectives all require isolation of the constituent and we may therefore assign cancer antigens to different categories depending on the ease with which a given constituent may be isolated and identified" (Rapport and Graf, 1961).

The discovery of nucleic acid was the result of work by Miescher (Jordon, 1960) who isolated in 1868 a substance that he called nuclein from pus cells. At this time he demonstrated the

highly polymeric character of nucleic acid, but unfortunately this was overlooked by later investigators. These workers using degradative methods of organic chemistry described the elementary constituents, but these analyses gave no hint concerning the structure of the biologically active molecule.

It wasn't until Avery et al. (1944) found that deoxyribonucleic acid (DNA) was the agent of bacterial transformation, that it was considered biologically active. Some years later Hershey and Chase (1952) found that DNA is the main component that enters the host bacterium from an infecting bacteriophage. Shortly after this Watson and Crick (1953) made proposals about the structure of DNA and its mode of replication. Since then much progress has been made concerning the structure and biological behavior of DNA.

With respect to cancer, nucleoproteins are of interest because they are the basic constituents of the chromatin structure of the cell nucleus. Considering the morphological irregularities in chromatin structure of the nucleus from the benign to malignant states, there appears to be a definite sequence of nuclear abnormalities. Histologically these have been well described. Koss and Durfee (1961) have reviewed this problem and have established morphologically a so-called "natural history of cancer." The series of events that lead to a carcinoma state, show nuclear abnormalities from hyperplasia, to metaplasia, to dysplasia and finally to the neoplastic product that fails to replicate itself as a functional cell.

Since the proposal by Watson and Crick (1953) concerning the structure of DNA, the trend of investigation has changed from morphological observations of chromatin structure of the cell nucleus, to the chemical and biological effects of nucleic acids. The Watson-Crick model of the structure of DNA furnished a concept regarding the possibility of self duplication of this type of biologic polymer. Since their original concept was enunciated, many investigators have considered that the specific DNA molecules contained in their nucleic acid sequence the "information" required for the biosynthesis of protein. This suggests then, that nucleic acids act as "templates" for protein biosynthesis. This concept implies that if nucleic acids in malignant cells are chemically different, the replication of protein produced by these abnormal nucleic acids would be different from their normal counterparts.

Chemically speaking some methods of measuring pyrimidines and purines show little difference between tumor and nontumerous tissues, Miller et al. (1950a, 1950b), Bloom et al. (1950). Woodhouse (1949) and Beal et al. (1950) reported considerable variation between the pyrimidines and purines in cancer tissue when compared to normal tissue. These differences reported between tissues appear to be based upon the analytical methods used and the means of degradation of the nucleoproteins.

Total content of nuclear DNA appears to vary with the type of tumor that is analyzed. Mark and Ris (1949) found that the DNA content

per nucleus of mouse hepatoma was similar to that of normal somatic cells. However, Klein et al. (1950) found a increased amount of DNA in Erhlich ascites tumor cells when compared with normal cells. Stich et al. (1960) demonstrated the variability of DNA content between normal epithelium, polyps and adenocarcinomas of the large intestines of man. These investigations support the statement of Schmidt (1959), that the intricate problems of nucleoprotein metabolism are all problems of the "chemical anatomy" of nucleic acids.

Evidence exists then to suggest that DNA from neoplastic cells in some instances appears to be different not only in amount, but also histologically and chemically when compared to normal cells. So far, in the utilization of DNA as an antigen, reports generally indicate poor antibody response, Lackman et al. (1940) and Phillips et al. (1958). The data reported by these latter groups suggested that DNA, isolated from bacteria, may contribute to the antigenic specificity. Miescher et al. (1959) using DNA prepared from cells of salmon sperm and calf thymus demonstrated an antibody response in rabbits although it appeared very weak. It is assumed from these experiments that DNA showed a serological specificity but the antigenic qualities were poorly demonstrated. It appears likely that if the molecular integrity of the DNA molecule could be maintained upon extraction that the antigenic qualities might be enhanced.

At present few reports are available concerning the possibility that DNA or DNP might be used to augment resistance to neoplastic

disease. Zilber (1958), because of his interest in the viral etiology of cancer presumed that nonfilterable tumors might still contain masked virus associated with nucleoproteins of the tumor. He isolated crude nucleoproteins from human and mouse tumors and instead of demonstrating oncogenic masked virus, found that the tumor nucleoproteins contained cancer-distinctive antigens. These antigens were demonstrated by immunizing guinea pigs and anaphylactic response was used as a measure of antigenicity. Recently Perez-Cuadrado et al. (1964a, 1964b) further substantiated the production of specific antiserum to human cancerous tissue by immunizing animals with cancer cell particles containing DNA-bound protein.

From previous data collected, Perez-Cuadrado et al. (1965a) tried to characterize cancerous tissue antigens by immunohistochemical and ultrastructural methods. Treating cancerous tissue with ultrasonic waves and by differential centrifugation, they separated the cellular components into 10 fractions. Each fraction was subsequently used for immunization of rabbits. Immunization of these animals led to the production of several antibodies, some of which were specific for human cancer antigens. The antibodies were demonstrated by agglutination of sensitized polystyrene latex particles. They found that the human cancer-specific antibodies were increased by immunizing antigens, which contained DNA-bound proteins from cancer cell nuclei. They further substantiated their findings (Perez-Cuadrado et al., 1965b), by testing the antibody produced by DNA-bound protein from

from cancerous tissue, using fluorescent antibody and agar gel double diffussion precipitation tests. From the evidence presented by these investigators, it is suggested that an immune response to neoplastic disease might be elicited by using unaltered DNA or DNP prepared from tumor tissue.

MATERIALS AND METHODS

I. TUMORS

A. Ehrlich Ascites Tumor (EAT), Ascites Form.

The Ehrlich ascites tumor has been described by Stewart et al. (1959) as "an undifferentiated tumor that originated spontaneously as a carcinoma of the mammary gland of a stock mouse." The tumor was originally obtained by Ehrlich in 1907 and has been carried through mouse to mouse passage for an unknown number of transfers. The strain of EAT used in the proceeding experiments was obtained from the Sloan-Kettering Institute and is one of several strains of EAT used in cancer research. Variation in the present day line is undoubtedly different from the original tumor and appears to vary not only in nutritional requirements, as observed in tissue culture, but also under different environmental circumstances of mouse passage. Stewart et al. (1959) have reported a variation in the number of chromosomes from 45-80. The tumor used in this laboratory has a modal chromosome number of 60, with a range from 41-83 (Oshiro, 1963). Furthermore, as described later in the section on experimental results, the LD_{50} of this tumor in Oshiro's experiments was different from the LD_{50} described in this report, although the tumor was obtained from the same stock.

B. Sarcoma 37 (S-37), Ascites Form.

Stewart et al. (1959) described this tumor as " a rapidly growing undifferentiated tumor that originated as a carcinoma of

the mammary gland of an untreated mouse." The tumor has been carried by mouse to mouse passage since 1906 and is not specific for any strain of mice. In many respects it is similar to EAT except for the LD_{50} which will be described later. The strain of S-37 tumor used in this laboratory was obtained from the National Institutes of Health and has been carried in this laboratory for seven years. Both S-37 and EAT were maintained in this laboratory by weekly transplants of 0.25 ml of ascites into albino mice.

II. ANIMALS

Albino mice (Mus musculus) of mixed sexes were used in all experiments. Before each experiment mice were picked at random and caged in 7" x 13" plastic cages in groups of 12 or less. They were acclimated for three days before each experiment. The animals were given a diet of commercially prepared food and water ad libitum.

III. TUMOR CELLS

Free tumor cells were obtained by withdrawing peritoneal exudate from tumor infected mice into heparinized physiologic saline (PSS) solution. The tumor cells were separated from the serosanguineous ascites fluid by centrifugation at 500 rmp for five minutes and decanting the supernatant fluid and red blood cells. The cells were "washed" three times in PSS, which resulted in a tumor cell suspension comparatively free from other cells.

Ehrlich and S-37 tumor cells were suspended in PSS and viable cells were counted in the hemocytometer by using a 0.05% solution of eosin in PSS as diluent. The unstained cells were considered viable. The cells were then diluted in PSS to the desired number. All injections were given in 1.0 ml amounts.

IV. PREPARATION OF DNP AND DNA

DNP and DNA were prepared employing modifications of the several methods reviewed by Jordan (1960). Sterile procedures were employed throughout and in order to inhibit enzymatic degradation of DNP and DNA, all procedures were carried out at 4°C. unless otherwise indicated.

A. DNP.

Thirty gram portions of the sedimented ascites tumor cells were washed three times in 0.9% NaCl and then blended in a high speed mixer for two minutes, with the addition of 20 ml of 1% NaCl, previously adjusted to pH7 by the addition of sodium bicarbonate solution. The sediment collected by centrifugation was again resuspended in 1% NaCl, mixed, and again centrifuged. To the sediment were added 50 ml of 10% NaCl, at pH7 and the suspension blended in the mixer for 15 seconds. The resulting viscous solution was then left standing and periodically stirred for one hour. The solution was poured into 300 ml of distilled water constantly agitated with a glass stirring rod and the resulting DNP precipitated into long viscous threads.

The DNP was collected by centrifugation and washed twice with distilled water. One gram portions of DNP were suspended in 6 ml of 0.9% NaCl with the addition of 5 units of penicillin and 10 micrograms of streptomycin to insure bacteriostasis. The material was then stored frozen at -20°C. until ready for use. Protein analysis of DNP prepared from different tumors in this laboratory yielded approximately 2.4 grams % protein, using the Biuret method for total protein (Henery, 1964).

B. DNA.

DNA was prepared by digestion of protein from DNP with trypsin. One hundred mg of crystalline trypsin (Miles Chemical Co., Clifton, New Jersey) was added to 6 gms of prepared DNP at Ph 7.4 and incubated at 37°C. for 30 minutes. With addition of 70% ethyl alcohol the digested material was allowed to sediment in the refrigerator at 4°C. The precipitate was washed twice in cold 70% ethyl alcohol and then 1 gm portions were dissolved in 6 ml of distilled water with the addition of 5 units of penicillin and 10 micrograms of streptomycin/ml to insure bacteriostasis. The material was stored frozen at -20°C. until used. Protein analysis from different DNA preparations in this laboratory yielded values of less than 1 mg% of protein using the Biuret method for total protein determination (Henery, 1964).

C. DNP From Normal Tissues.

Liver, heart, lung and kidneys were removed from mice and DNP prepared in the same manner as described for tumor cells.

V. IMMUNIZATION

Mice were injected intraperitoneally with 25 mg of DNP. Four injections were given with an interval of seven days between each injection. A similar immunization schedule was carried out using DNA, except the amount of inoculum was 10 mg. After the last injection, there was a thirty-day interval before challenge.

VI. PERITONEAL WASHINGS

Peritoneal washings for microscopic observation of immune response were made at various time intervals. Mice were sacrificed and the peritoneal cavity washed with PSS. The collected fluid was centrifuged and wet and fixed smears made from the sedimented cells.

VII. STAINING METHODS

Two different stains were used for examination of cells; a supravital stain for the differentiation of histiocytes and leucocytes from neoplastic and mesothelial cells in the ascites fluid and a modified Papanicolaou's stain for fixed cells.

A. Papanicolaou Stain and Procedure. (Koss and Durfee, 1961)

1. Wet smears on glass slides were fixed in 95% ethyl alcohol and ether, 1:1 for 20 minutes. The slides were then treated in staining jars as follows:

2. 80% ethyl alcohol - 10 dips
3. 70% ethyl alcohol - 10 dips
4. 50% ethyl alcohol - 10 dips
5. Water - 10 dips
6. Harris hemotoxylin - 2½ minutes
7. 1% hydrochloric acid in 70% ethyl alcohol - 5 dips
8. Running tap water - 5 minutes
9. 50% ethyl alcohol - 10 dips
10. 70% ethyl alcohol - 10 dips
11. 80% ethyl alcohol - 10 dips
12. 95% ethyl alcohol - 10 dips
13. Orange G (stain) - 1½ minutes
14. 95% ethyl alcohol - 10 dips
15. 95% ethyl alcohol - 10 dips
16. EA 50 (stain) - 1½ minutes
17. 95% ethyl alcohol - 10 dips
18. 95% ethyl alcohol - 10 dips
19. 95% ethyl alcohol - 10 dips
20. Isopropyl alcohol - 10 dips
21. Xylol - 5 minutes
22. Slide preserved with Permount and cover slip

B. Supravital Staining (Koss and Durfee, 1961).

Saturated solutions of neutral red and Janus green in absolute ethyl alcohol were prepared in separate well stoppered bottles. Dilute solutions were then made from each of these by adding 20 to 50 drops of neutral red stock solution or 15 to 30 drops of the

saturated solution of Janus green to 10 ml of absolute alcohol. For use, 10 - 50 drops of the dilute Janus green solution were added to 2 ml of the dilute neutral red. A drop or two of this mixture was placed on a clean slide, another slide inverted over this wet stain and the two slides drawn apart so that an evenly distributed film was left on one side of either slide. For use, cellular material was smeared on the dry film of the stain. Janus green stains the lymphocytes and neutral red stains the granules of the histiocytes and also the polymorphonuclear leucocytes. Neoplastic or mesothelial cells, with few exceptions, did not stain by this method.

EXPERIMENTAL RESULTS

I. LD_{50} DETERMINATION

It has been shown that greater accuracy in measuring immune response, and better economy in the use of animals can be exercised, if a dose can be determined which will kill 50% of the animals under test. This dose is better known as the LD_{50} (lethal $dose_{50}$). If this value can be determined using variable numbers of viable tumor cells, then gradations of numbers of cells used for challenge in an immunized animal would be more meaningful. This value would also tend to establish a base line of reference for the study of degrees of resistance to tumor cell challenge. The hypothesis is set forth that a critical number of tumor cells might cause disease, while a lesser number would fail to induce tumor in the average animal.

In order to test this hypothesis, known numbers of tumor cells were injected intraperitoneally into albino mice using strain-non-specific ascites tumors. The tumors employed throughout this study were EAT and S-37 mouse tumor cells. Although these two tumors were indistinguishable morphologically, there was a striking difference in the number of cells needed for infectivity. The results shown in Tables 1 and 2 and Figures 1 and 2 indicate that the LD_{50} for EAT was approximately 10^3 cells, while that of S-37 was 5×10^4 cells. Not apparent from these tables is that the cause of death in animals challenged with both tumors appeared to be increasing ascites as histological sections showed no metastases to other organs.

Table 1. Mortality ratios following challenge with Ehrlich ascites tumor cells by the intraperitoneal route with LD_{50} value*

Challenge Dose	Mortality	% Mortality
10^1	0/20	0%
10^2	3/21	14%
10^3	7/22	31%
10^4	15/23	65%
10^5	22/22	100%

Numerical LD_{50} and 95% confidence limits = $2,000 \pm 560$

* Data analyzed by the method of Miller and Taintor (1944)

Figure 1. Probit transformation of mortality data following challenge of white mice with EAT cells by intraperitoneal route.

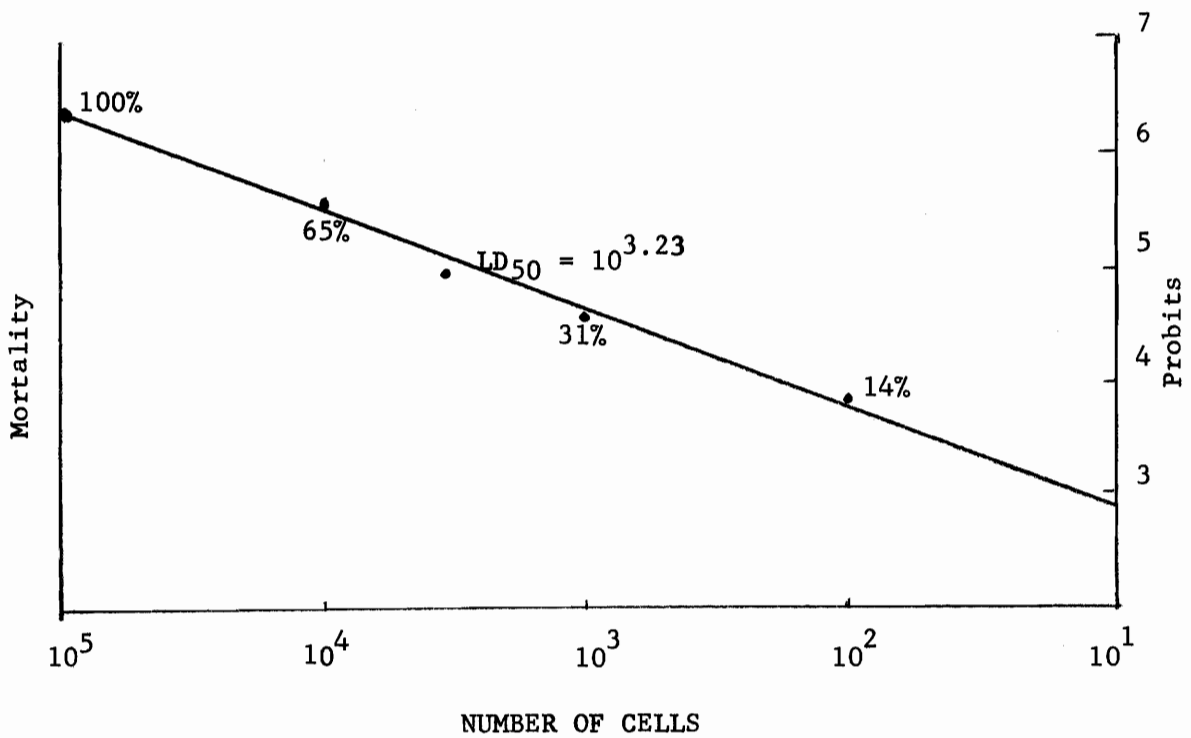
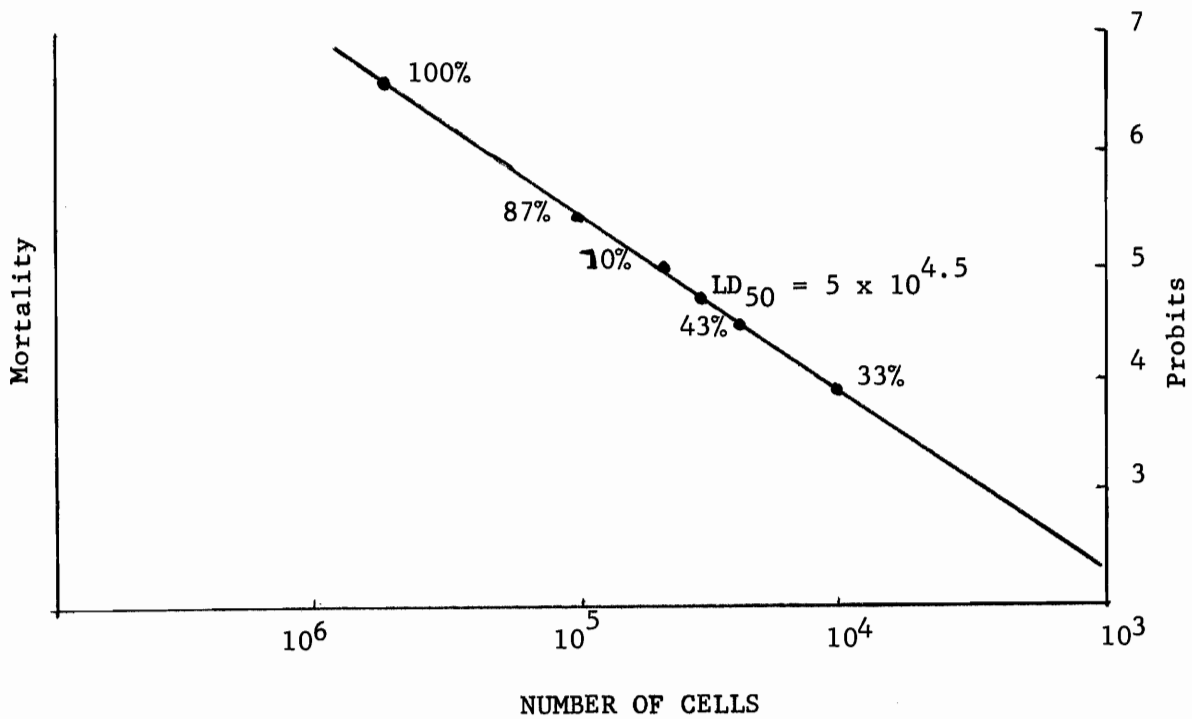


Table II. Mortality ratios following challenge with S-37 tumor by intraperitoneal route. LD_{50} value.

Challenge Dose	Mortality	% Mortality
10^1	0/23	0%
10^2	0/22	0%
10^3	0/22	0%
10^4	3/23	13%
$3x10^4$	8/24	33%
$5x10^4$	10/23	43%
$1x10^5$	15/22	70%
$2x10^5$	20/23	87%
$4x10^5$	100/100	100%

Numerical LD_{50} and 95% confidence limits = $45,000 \pm 11,866$

Figure II. Probit transformation of mortality data following challenge of white mice with S-37 cells by intraperitoneal route.



II. ABSORPTION SPECTRUM OF DNA

Purity of the prepared DNA from EAT and S-37 tumor cells was compared with a known purified standard of DNA (Sigma Chemical Co., St. Louis, Mo.). The instrument used was the Beckman D U spectrophotometer. The ultraviolet absorption curves of the prepared and standard DNA in distilled water were compared with each other. The resultant curves, as shown in Figure III. indicated a high degree of purity of the prepared material.

III. IMMUNOLOGICAL RESPONSE TO DNP AND DNA

The hypothesis has been formulated that "cancer specific" antigens do exist and that these antigens can induce an immune response. The operational approach suggested was that by using essentially unaltered preparations, (Jordon, 1960) the prepared material would retain its antigenic identity. If this premise is true, then the antigenic capabilities of DNA or DNP would be increased and competitive antigenic inhibition, which is induced by whole cell homogenates, would be minimized. In order to test this hypothesis the following experiments were performed.

In the routine transfer of EAT cells in control animals, death regularly occurred in one-half of the animals inoculated when approximately 10^3 EAT cells were injected intraperitoneally. In order to evaluate the response in immunized animals over a wider range, challenge of 10^2, 10^3, 10^4 and 10^5 cells/animal were performed.

Similarly S-37 cells have an LD_{50} of approximately 50,000 cells and immune response was evaluated over a range of $5x10^4$, 10^5, $2x10^5$ and $4x10^5$ viable cells. Groups of 24 mice were employed for each dose. Each experiment was terminated after 50 days, and the per cent living was compared to the per cent dead. A summation of the experimental design is shown in Figure IV.

Table III shows the results obtained when mice immunized with DNP from EAT cells were compared to control animals; animals in both groups were challenged with varying numbers of viable EAT cells. Similarly, Table IV summarizes results obtained with mice immunized with DNP from S-37 cells and the control group; both groups were challenged with varying numbers of viable S-37 cells. The results supported the working hypothesis that a dose-response effect is apparent with both normal and immunized groups. Table III shows that in one instance 86% of the immunized animals survived as compared to 100% mortality in the control animals. Although the degree of immunity was not as great in the S-37 experiment, nevertheless, 50% of the animals were protected as compared to 96% mortality in the control animals with a challenge dose of $4x10^5$ viable tumor cells.

To test for the existance of cross immunity between DNP from EAT cells and S-37 cells, mice were immunized with DNP of one tumor and challenged with cells of the other. The results are shown in Tables V and VI. The results demonstrated that mice immunized with DNP from EAT cells and challenged with S-37 cells were protected

Figure III. Absorption Spectrum of DNA

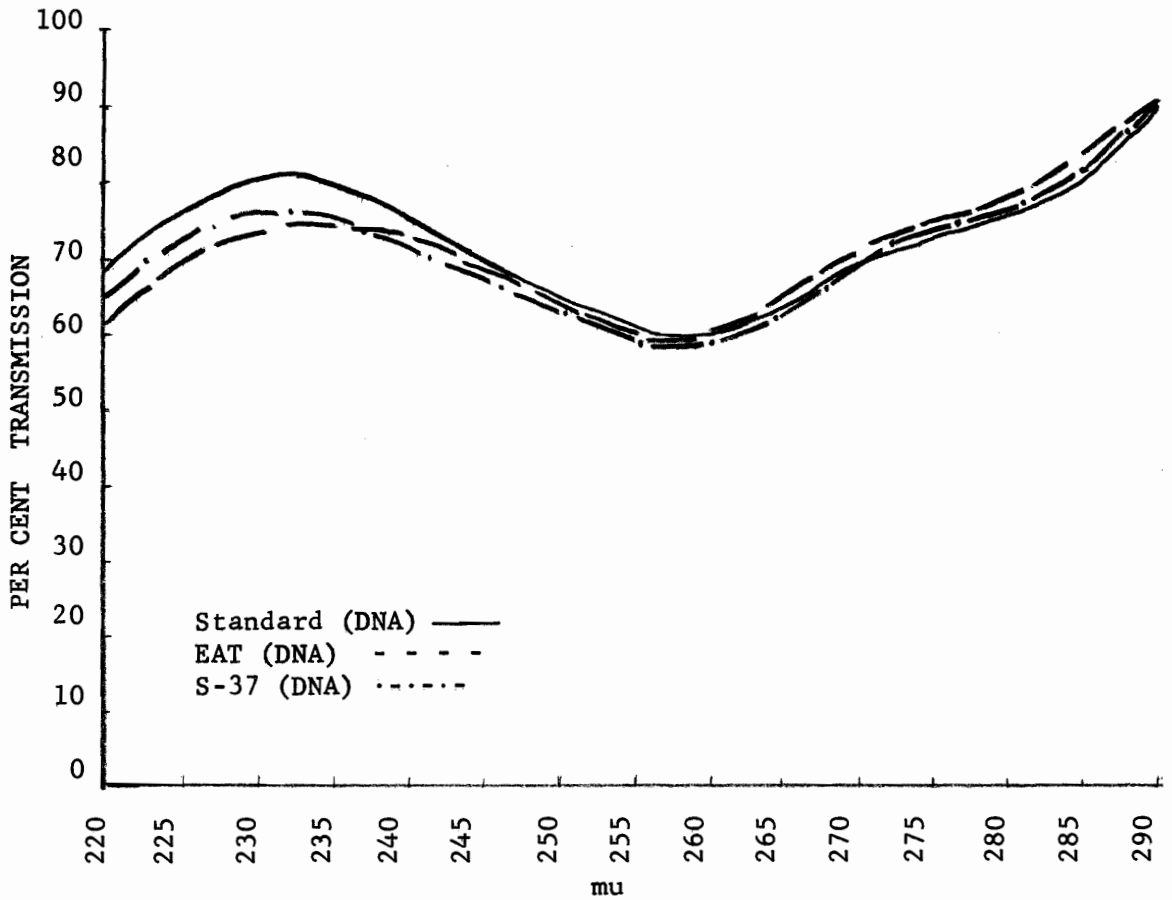


Figure IV. Summation of Experimental Design

Mice Immunized with	Mice Challenged with
DNP from EAT	EAT cells
DNP from S-37	S-37 cells
DNP from EAT	S-37 cells
DNP from S-37	EAT cells
DNA from EAT	EAT cells
DNA from S-37	S-37 cells
DNA from EAT	S-37 cells
DNA from S-37	EAT cells
DNP from normal tissue	S-37 cells

Table III. Comparison between mice immunized with DNP from EAT cells and the control group (saline treated) challenged with varying numbers of viable EAT cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
10^2	0/20	0%	0/23	0%	--
10^3	0/21	0%	5/24	13%	.10
10^4	0/21	0%	16/24	67%	<.001
10^5	3/22	14%	22/22	100%	<.001

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table IV. Comparison between mice immunized with DNP from S-37 cells and the control group (saline treated) challenged with varying numbers of viable S-37 cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
$5x10^4$	6/22	27%	10/23	43%	.5 - .3
$1x10^5$	5/23	22%	13/23	56%	.05 - .02
$2x10^5$	7/23	30%	22/24	87%	<.001
$4x10^5$	11/22	50%	23/24	96%	.001

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table V. Comparison between mice immunized with DNP from EAT cells and the control group (saline treated) challenged with a varying number of viable S-37 cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
$5x10^4$	4/21	19%	10/23	43%	.2 - .10
$1x10^5$	6/22	27%	15/24	62%	.05 - .02
$2x10^5$	7/21	33%	20/24	83%	.001
$4x10^5$	11/23	48%	24/24	100%	.001

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table VI. Comparison between mice immunized with DNP from S-37 cells and the control groups (saline treated) challenged with a varying number of viable EAT cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
10^2	0/22	0%	0/23	0%	--
10^3	0/23	0%	5/24	13%	.1
10^4	5/23	21%	16/24	67%	.01
10^5	10/24	47%	24/24	100%	<.001

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table VII. Comparison between mice immunized with DNA from EAT cells and the control group (saline treated) challenged with varying number of viable EAT cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
10^2	0/16	0%	2/20	10%	.2
10^3	0/16	0%	9/20	45%	.01
10^4	0/16	0%	15/20	75%	<.001
10^5	6/16	37%	20/20	100%	<.001

*Chi square analysis with Yates' correction. Significant difference values underlined.

to the same extent as when DNP from S-37 cells was used as the immunizer. However, when mice immunized with DNP from S-37 were challenged with EAT cells, a lesser degree of protection was demonstrated as compared to immunization with DNP from EAT. The results do indicate that although protection was diminished in this latter instance that a cross immunity between these two tumors did exist.

Since immunity as expressed in the foregoing experiments was greater than anticipated, it was desirable to determine if the protein moiety of the DNP was necessary for immunogenicity. Protein was removed from the DNP as described in the Materials and Methods section. The results shown in tables VII and VIII support the premise that purified DNA retained its antigenicity even though protection was diminished as compared to DNP. This was particularly evident with mice immunized with S-37 DNA. However, as was demonstrated in the previous experiment S-37 DNP did not afford as high protection level against challenge with that tumor as did the EAT preparations against the EAT tumor.

In order to test if the protein moiety of DNA may in some way contribute to cross immunity of EAT and S-37 tumors, purified DNA from EAT cells and S-37 cells were used to immunize mice. The immunized mice were then challenged with S-37 or EAT cells. The results shown in Tables IX and X indicate that cross immunity would still be demonstrated, however, to a considerably lesser degree.

Table VIII. Comparison between mice immunized with DNA from S-37 cells and the control group (saline treated) challenged with varying number of viable S-37 cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
$5x10^4$	8/20	40%	11/20	55%	.5
$1x10^5$	11/20	55%	15/20	75%	.5 - .3
$2x10^5$	18/20	90%	20/20	100%	.5 - .3
$4x10^5$	16/20	80%	20/20	100%	.2

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table IX. Comparison between mice immunized with DNA from EAT cells and the control group (saline treated) challenged with varying number of viable S-37 cells.

Challenge	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
$5x10^4$	5/20	25%	11/20	55%	.1
$1x10^5$	5/19	26%	15/20	75%	.01
$2x10^5$	11/18	61%	20/20	100%	.01
$4x10^5$	18/20	90%	20/20	100%	.5

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table X. Comparison between mice immunized with DNA from S-37 cells and the control group (saline treated) challenged with varying number of viable S-37 cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
10^2	0/19	0%	2/20	10%	.5 - .3
10^3	2/19	10%	9/20	45%	.05 - .02
10^4	1/20	5%	15/20	75%	.001
10^5	14/20	70%	20/20	100%	.05 - .02

*Chi square analysis with Yates' correction. Significant difference values underlined.

IV. IMMUNOLOGICAL RESPONSE TO DNP PREPARED FROM NORMAL TISSUE

It has previously been stated that tumor cells are not only morphologically different (Koss and Durfee, 1961) but also chemically different (Mark and Ris, 1949 and Beal et al., 1950) from normal cells. Thus, if cancer distinctive antigens exist, they would not normally be demonstrated in non-cancerous cells. To test if an immune response might be elicited by DNP from normal cells, mice were immunized with DNP prepared from normal mouse tissue and subsequently challenged with EAT and S-37 cells. The results shown in Tables XI and XII supported the original hypothesis, i.e., immunological response to injection of DNP from normal tissue cells was not protective against challenge by either S-37 or EAT.

V. OBSERVATION OF IMMUNE RESPONSE

In order to visualize microscopically the immune response, 24 mice immunized with DNP from S-37 were challenged intraperitoneally with $4x10^5$ viable S-37 cells. At various time intervals the peritoneum was washed with 1 ml of PSS, and the cells were collected for microscopic study. A wet mount and permanent preparations were made with material from each animal in both the immunized and control groups. Two animals from each group were sacrificed at the given time intervals. The observations are listed in chronological order.

Table XI. Compairson between mice immunized with DNP from normal mouse tissue and the control group (saline treated) challenged with a varying number of viable EAT cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
10^2	0/11	0%	1/12	8%	> .99
10^3	4/11	34%	5/12	41%	> .80
10^4	8/12	66%	7/11	63%	> .90
10^5	11/11	100%	10/10	100%	---

*Chi square analysis with Yates' correction. Significant difference values underlined.

Table XII. Comparison between mice immunized with DNP and normal mouse tissue and the control group (saline treated) challenged with a varying number of viable S-37 cells.

Challenge Dose	Immunized	Per Cent Mortality	Control	Per Cent Mortality	P Value*
$5x10^4$	6/12	50%	5/12	41%	$\rangle .99$
$1x10^5$	6/11	54%	7/11	63%	$\rangle .99$
$2x10^5$	9/10	90%	10/12	83%	$\langle .80$
$4x10^5$	11/12	91%	11/11	100%	$\rangle .99$

*Chi square analysis with Yates' correction. Significant difference values underlined.

In order for the three different types of cells, tumor, lymphocytes and histiocytes to be distinguished, an enlargement was made, as is shown in Figure V, in order to classify the cells in the following photomicrographs.

One Hour Sampling.

Figure VI. Immunized. There appeared to be gross aggregates of tumor cells showing degeneration and a marked infiltration of lymphocytes and histiocytes.

Figure VII. Control. Tumor cells appeared uniformly dispersed and very few lymphocytes and histiocytes were noted.

Two Hour Sampling.

Figure VIII. Immunized. Relatively few tumor cells were observed but a marked lymphocytic and histiocytic response was still present.

Figure IX. Control. Tumor cells were moderate in number and few lymphocytes or histiocytes were seen.

Eighteen Hour Sampling.

Figure X. Immunized. There appeared to be only a few tumor cells with a marked lymphocytic and histiocytic response.

Figure XI. Control. Marked numbers of tumor cells, appearing as sheets and aggregates were noted. Within the aggregated tumor cells some appeared which seemed to be histiocytes, otherwise the histiocytic response was slight.

TWENTY-FOUR HOUR SAMPLING.

Figure XII. Immunized. Only a few scattered tumor cells were seen with rare clumps of cells that appeared to be lysing. The histiocytes and lymphocytes were still abundantly present.

Figure XIII. Control. Marked numbers of tumor cells and aggregates were observed with a diffuse, marked increase in lymphocytes and some histiocytes.

FORTY-EIGHT HOUR SAMPLING.

Figure XIV. Immunized. Only a rare tumor cell was noted among many lymphocytes and histiocytes.

Figure XV. Control. Massive numbers of tumor cells with many lymphocytes and histiocytes were observed.

SEVENTY-TWO HOUR SAMPLING.

Figure XVI. Immunized. Only a rare degenerating tumor cell was observed among many lymphocytes and histiocytes.

Figure XVII. Control. Massive numbers of tumor cells with few lymphocytes and histiocytes were observed.

These results support the hypothesis of Baker et al. (1962), and Bennett (1965) that immune destruction of ascites tumor cells is due largely to the contact between peritoneal inflammatory cells, histiocytes, and lymphocytes and tumor cells. If should be noted that the immune animals exhibited an immediate increase of cellular elements, while the control animals showed a 24-hour delay before histiocytes

and lymphocytes became prominent. Furthermore, there was a steady decrease in the numbers of tumor cells in the immune animal while the unimmunized animals showed a marked and overwhelming number of tumor cells after 24 hours. It is interesting to note that only after many tumor cells were observed in the control animals were histiocytes observed in any number.

PHOTOMICROGRAPH OF TUMOR CELL, LYMPHOCYTE AND HISTIOCYTE

Figure V. Enlargement of the three types of cells that are shown in the following photomicrographs. Tumor cell (A), lymphocyte (B) and histiocyte (C).

ONE HOUR SAMPLING

Immune

(X100)

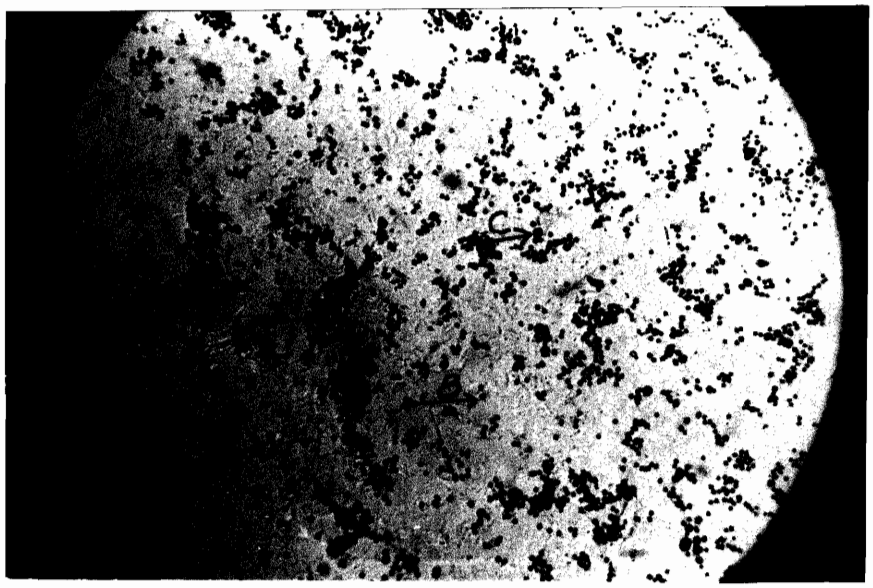


Figure VI. Aggregates of tumor cells (A) and marked infiltration of lymphocytes (B) and histiocytes (C).

Control

(X100)

Figure VII. Tumor cells (A) uniformly dispersed; few lymphocytes (B) and histiocytes (C).

TWO HOUR SAMPLING

Immune

(X100)

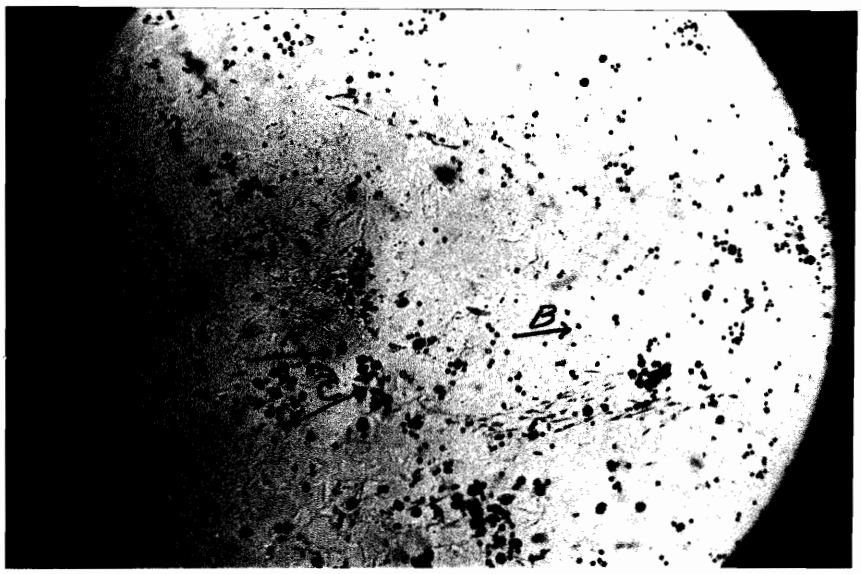


Figure VIII. Relatively few tumor cells (A); marked lymphocytic (B) and histiocytic (C) response.

Control

(X100)

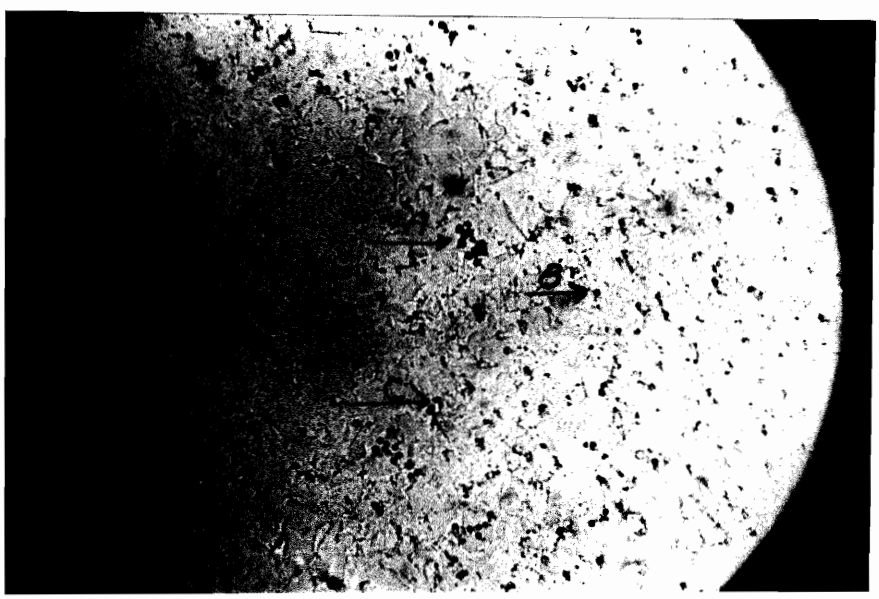


Figure IX. Tumor cells (A) are moderate in number; few lymphocytes (B) or histiocytes (C) are seen.

FORTY-EIGHT HOUR SAMPLING

Immune

(X100)

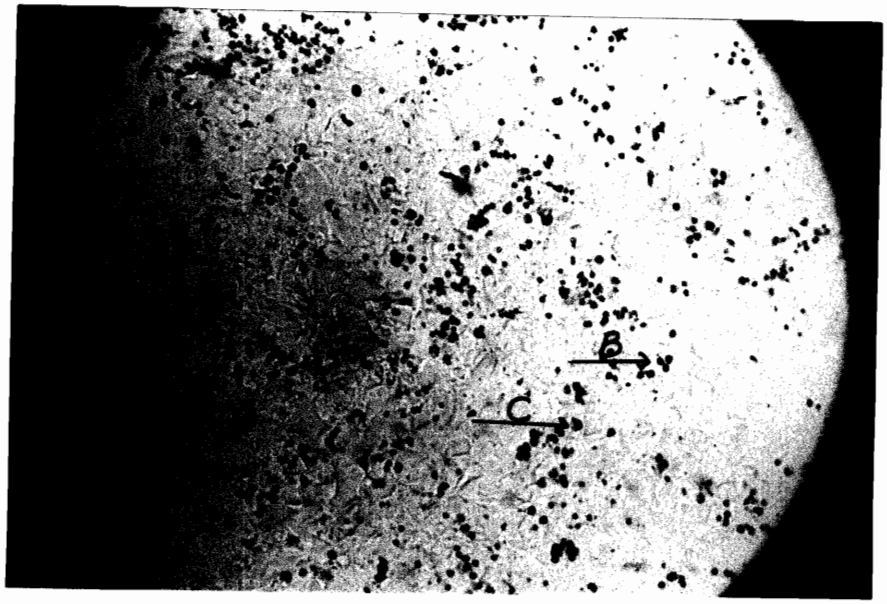


Figure XIV. Tumor cells (A) rare among many lymphocytes (B) and histiocytes (C).

Control

(X100)

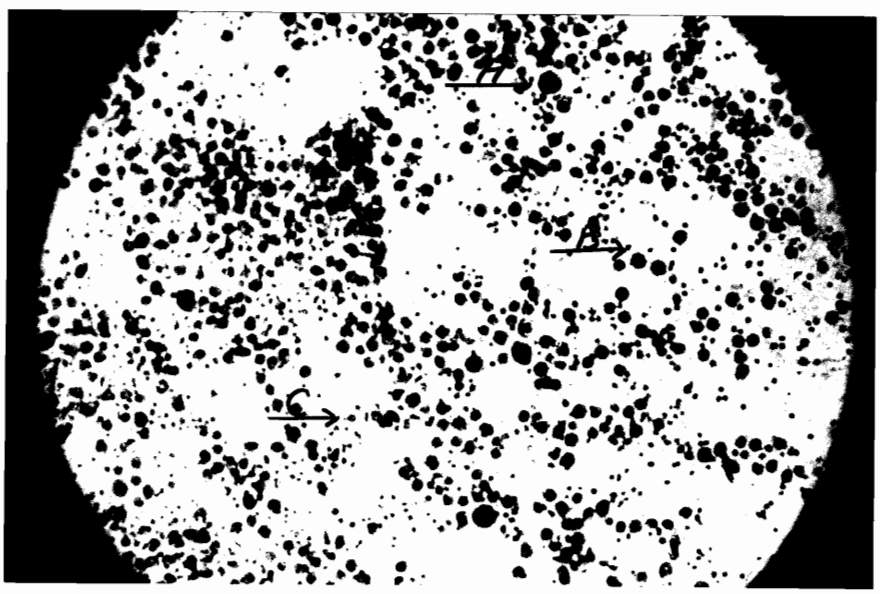


Figure XV. Massive numbers of tumor cells (A) with many lymphocytes (B) and histiocytes (C) present.

SEVENTY-TWO HOUR SAMPLING

Immune

(X100)

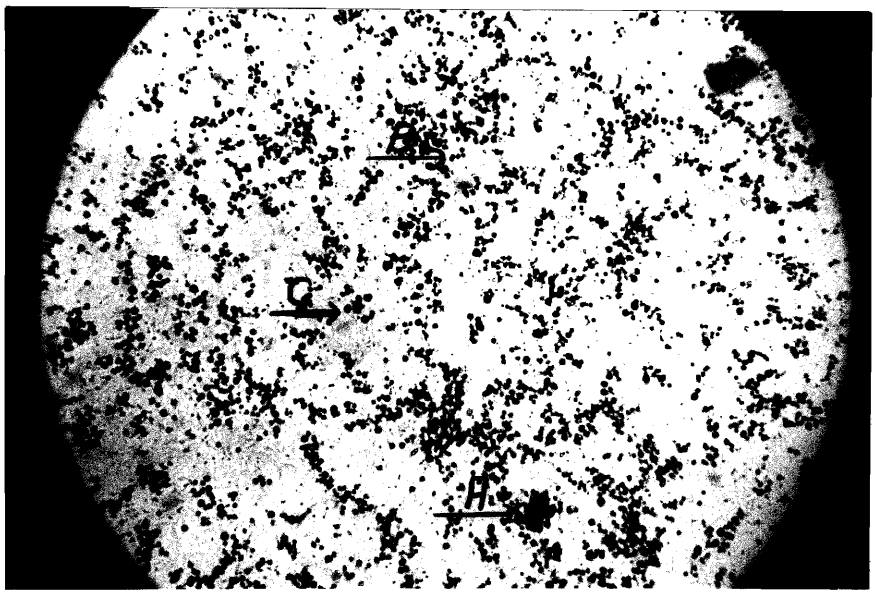


Figure XVI. Only rare, degenerating tumor cells (A) observed among many lymphocytes (B) and histiocytes (C).

Control

(X100)

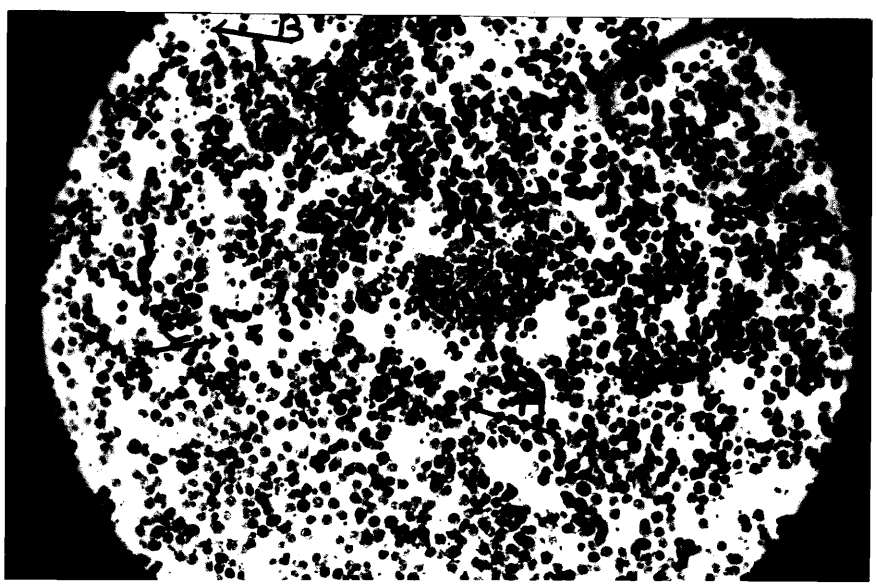


Figure XVII. Massive numbers of tumor cells (A) with few lymphocytes (B) and histiocytes (C) present.

DISCUSSION

The work that has been presented has involved the search for and characterization of a tumor specific antigen. The antigens DNP and DNA from tumor cells were employed to test the hypothesis that these antigens, so defined, could be used to augment resistant to neoplastic disease. Several conclusions can be made from the experimental results. These are: the establishment of an LD_{50} and the standard error of this value, in terms of cell numbers for experimentally transmitted neoplasms; that cancer specific antigens can be prepared from tumor cells, and there is enhancement of the immune response to cancer by the use of these antigens; there exists a cellular and probably a humoral resistance mechanism.

The LD_{50} values of EAT and S-37 tumor cells was noticeably different, even though the tumor cells are morphologically similar to each other. This implied that cells derived from EAT better resisted the influence of natural resistance mechanisms as they existed. However, once the tumor process was established, the outcome of the disease always ended in death of the animal. It was established, at least through the course of these experiments, that the LD_{50} values remained stable. Any descrepancies varied within the standard error. Although the tumors used were derived from the same stock as Oshiro's (1963), who established infectivity with EAT as less than 10 cells, in the work reported in this thesis, LD_{50} values of greater than 1,000 cells were consistently obtained. The

basis for the difference in LD_{50} values in these two reports are purely speculative. The difference in LD_{50} values may be accounted for in the time factor between processing these cells to the desired concentration and injection time, which might affect the viability of the cells. Another possibility might be that the diluent used for cells in these experiments was PSS rather than Hanks balanced salt solution as in Oshiro's experiments. This might also affect the viability of the cells.

The LD_{50} of S-37 tumor appeared to be more consistant with Oshiro's (1963) results. He reported an LD_{50} value of 25,000 $\pm$ 8,928. The results collected in this work and given in Table II were within that limit.

The immune mechanisms that are operative in the establishment of an LD_{50} value for these models were not investigated. However, it may be deduced that since a dose-response effect existed and an LD_{50} value may be determined, some type of resistance must exist normally. That is, a normal resistance mechanism exists for this model of a mammalian neoplastic disease. The existance of the LD_{50} value in normal animals was employed as the basis for the study of enhanced resistance.

By employing modifications of several methods described by Jordon (1960), the prepared DNP and DNA remained essentially unaltered. With the use of material that still retained its "chemical anatomy", it is believed that the intact molecules could be recog-

nized as a specific antigenic substance which differed from normal cell constituents. This is consistent with Landsteiner's (1946) work in which it was demonstrated that simple side chains of organic compounds could be recognized as imparting antigenic specificity. The purity of DNA was established by ultraviolet absorption with a known standard. The purity of DNP was not assayed because of the insolubility of this substance; it was assumed, however, from the results obtained after the removal of the protein moiety from the DNP molecule that DNP was a relatively pure preparation.

The results, using DNP as the immunizing agent, indicated that substantial protection was afforded against challenge by viable tumor cells. This is particularly true using DNP derived from EAT cells. Mice immunized with S-37 DNP showed considerably less protection; however, the mortality figures still support the fact that immunity can be established using this preparation. These results support Zilber's (1958) findings demonstrating an antibody to DNP. This latter investigation demonstrated DNP antigenic capacity by anaphylaxis and not by any protective measure that might be elicited using challenge tests. Zilber's (1958) findings are in contrast to some reports indicating that preparations of nuclear materials exhibited weak antibody response (Lackman et al., 1940 and Phillips et al. 1959). Zilber's methods of extraction were apparently less denaturing than previous workers.

The fact that immunization with DNP prepared from one tumor

followed by challenge with tumor cells of another resulted in protection might be interpreted to mean that the original hypothesis that cancer cells *per se* contain specific antigens is not tenable. However, DNP from normal tissue was not protective and the results obtained under the circumstances of the experimental designs described indicate that DNP from these two different tumors contain mutual antigens.

Results of similar experiments involving DNA without its protein moiety further supports the concept that the antigenic capabilities must reside in the molecular arrangement of DNA from cancer cells. Although demonstration of protection as tested by challenge was quantitatively less than when DNP was used for immunization, protection was still enhanced although to a lesser degree. This is consistent with the fact demonstrated by Landsteiner (1946), that the antigenic specificity resides in the molecular arrangement of simple organic compounds but that this capacity is demonstrable only when the simple compounds were first coupled to a protein carrier.

The experiments involving DNP prepared from normal mouse tissue yielded significant results. That is, this material provided no protection as an immunizing agent. When mice immunized according to the standard schedule were tested by challenge with tumor cells, no protection was noted either measured as decreased mortality or increased longevity. This finding furthers the concept of the existence of tumor specific antigens.

There are available at present few reports concerning the possible role of macrophages in resistance to neoplastic disease. Amos (1960) reported active ingestion and digestion of E.L. 4 and L1210 tumor cells by histiocytes from C3H mice *in vivo* as well as a direct antagonistic effect of histiocytes on MCLM tumor cells. Similarly, Weaver (1958) described the role of macrophages in ingestion and intracellular distruction of DBA/2 thymus tumor cells in ascites form in C57 BL/6 mice. Microscopic examination of the cellular response made by observing tumor cells and macrophages from peritoneal washings of normal and immunized mice substantiated the observations of Baker *et al*. (1962) and Linderman (1964). The experimental data of these latter investigators demonstrated that the initial defence mechanism primarily concerned macrophages. However, Linderman (1964) also demonstrated that serum or plasma from immune mice could transfer immunity to challenge by the original viable tumor. Our observations included not only an initial influx of macrophages and lymphocytes in the immune animal, but also what appeared to be lysis of some viable tumor cells. Thus, it was assumed from this limited experience that the defense mechanisms involved were not only cellular but also humoral. Further experimentation would be needed to substantiate these observations.

SUMMARY

1. LD_{50} values and 95% confidence limits were determined for the ip route with one strain each of EAT and S-37 tumors. The values obtained were: for EAT 2,000 $\pm$ 560; for S-37 45,000 $\pm$ 11,866.
2. DNP and DNA were prepared from EAT and S-37 tumors and the prepared material was used for immunization.
3. Mice immunized with DNP prepared from EAT and S-37 and subsequently challenged with viable EAT and S-37 tumor cells showed a considerable degree of resistance as compared to the unimmunized control animals.
4. Cross immunity was demonstrated between mice immunized with DNP from one tumor and challenged with tumor cells other than the type used for immunization.
5. Mice immunized with DNA prepared from EAT and S-37 cells and challenged with viable EAT and S-37 tumor cells showed increased resistance as compared to the unimmunized control animals. The degree of resistance induced using material without the protein moiety was less than that induced by using DNP.
6. Cross immunity was also demonstrated between mice immunized with DNA from one type of tumor cell and then challenged with tumor cells other than the type from which the nucleic acid for immunization was obtained. However, the immunity in this latter circumstance was considerably less than when the immunization was carried out with the corresponding DNP.

7. DNP prepared from normal mouse tissue and used for immunization showed no measurable induction of resistance when the mice were tested by challenge with viable tumor cells.
8. Microscopic observation of peritoneal washings from immunized and normal mice that had been challenged with viable tumor cells showed a significantly different cellular and humoral response over a 72-hour period. This response was indicated by lymphocytic and histiocytic infiltration and by lysis of tumor cells.

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RESEARCH PROPOSALS

submitted

by

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degree of

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RESEARCH PROPOSALS

1. Studies have indicated that DNA from neoplastic cells contain cancer specific antigen(s). Would recombination of DNA from different types of cancerous tissue increase the antigenicity?

2. It has been found that if solutions of DNA from Escherichia coli and Shigella dysenteriae were heated together and cooled slowly they formed some hybrid double stranded molecules with one strand from each type of DNA. By combining DNA from different viruses, can antigenicity be increased?

3. Critical, properly designed investigation should be undertaken to determine whether DNP prepared from cancer tissue removed at surgery can be used to augment resistance to the neoplastic process in humans.

4. Since ribosomes are the final agents in protein synthesis which is mediated by messenger RNA from DNA, does ribosomal RNA contain a cancer specific antigen?

5. Some strains of mice develop mammary carcinoma which is transmitted by the "milk factor" from parent to offspring. Could these mice be protected from developing carcinoma by immunizing with DNP from these solid tumors?

6. Since DNA appears to be antigenically specific in cancer tissue, would DNA from normal tissue exhibit antigenicity in allogenic experiments of tissue transplantation? If antigenicity could be thus demonstrated, probable enhancement of the transplanted tissue might be induced.

7. Recent experiments have indicated that tumor rejection is primarily cellular in nature. Undoubtedly humoral mechanisms are also involved. Do these two immunological events act independently or are they mutually dependent upon one another?

8. Adoptive transfer is carried out by transfer of immunologically competent cells from an immune to a non-immune individual. Would extraction of DNP from this cell achieve the same results?

9. There are at present many drugs used in the treatment of carcinoma. Could the survival rate be increased by using mice immunized with DNP in conjunction with anti-cancer drugs?

10. Karyotype analysis involves culturing lymphocytes of the blood; could the same results be achieved by abrading the skin and placing glass coverslips over this area?

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